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<p>(21) International Application Number: <b>PCT/US96/08086</b></p> <p>(22) International Filing Date: 3 June 1996 (03.06.96)</p> <p>(30) Priority Data:</p> <table> <tr> <td>08/473,544</td> <td>7 June 1995 (07.06.95)</td> <td>US</td> </tr> <tr> <td>08/477,725</td> <td>7 June 1995 (07.06.95)</td> <td>US</td> </tr> </table> <p>(71) Applicant (<i>for all designated States except US</i>): ALKER-MES CONTROLLED THERAPEUTICS, INC. [US/US]; 4th floor, 64 Sidney Street, Cambridge, MA 02139 (US).</p> <p>(72) Inventors; and</p> <p>(75) Inventors/Applicants (<i>for US only</i>): JOHNSON, OluFunmi, Lily [NG/US]; 10 Centre Street, No. 1E, Cambridge, MA 02139 (US); GANMUKHI, Medha, M. [IN/US]; 426 Staghorn Drive, Wexford, PA 15090 (US). BERNSTEIN, Howard [CA/US]; 929 Massachusetts Avenue, No. 2B, Cambridge, MA 02139 (US). AUER, Henry [US/US]; 130 Waverly Street, No. 1, Belmont, MA 02178 (US). KHAN, M., Amin [GB/US]; 29 Twin Pines Road, Dowington, PA 19335 (US).</p> <p>(74) Agents: BROOK, David, E. et al.; Hamilton, Brook, Smith &amp; Reynolds, Two Militia Drive, Lexington, MA 02173 (US).</p>		08/473,544	7 June 1995 (07.06.95)	US	08/477,725	7 June 1995 (07.06.95)	US	<p>(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</p> <p>Published <i>Without international search report and to be republished upon receipt of that report.</i></p>	
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(54) Title: COMPOSITION FOR SUSTAINED RELEASE OF HUMAN GROWTH HORMONE

## (57) Abstract

A composition, and methods of forming and using said composition, for the sustained release of biologically active, stabilized human growth hormone (hGH). The sustained release composition of this invention comprises a polymeric matrix of a biocompatible polymer and particles of biologically active, stabilized hGH, wherein said particles are dispersed within the biocompatible polymer. The method of the invention for producing a composition for the sustained release of biologically active hGH, includes dissolving a biocompatible polymer in a polymer solvent to form a polymer solution, dispersing particles of biologically active, metal cation-stabilized hGH in the polymer solution, and then solidifying the polymer to form a polymeric matrix containing a dispersion of said hGH particles. The method for using a composition of the invention is a method for providing a therapeutically effective blood level of biologically active, non-aggregated hGH in a subject for a sustained period. In this method, a subject is administered an effective dose of the sustained release composition of the present invention. The method of using the sustained release composition of the present invention comprises providing a therapeutically effective blood level of biologically active, non-aggregated human growth hormone in a subject for a sustained period by administering to the subject a dose of said sustained release composition.

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COMPOSITION FOR SUSTAINED RELEASE OF  
HUMAN GROWTH HORMONE

Background of the Invention

5 Human growth hormone (hGH) is a protein secreted by the pituitary gland and which can be produced by recombinant genetic engineering. hGH will cause growth in all bodily tissues which are capable of growth.

10 hGH is typically used to treat patients suffering from hypopituitary dwarfism. Currently, aqueous hGH is administered as a subcutaneous bolus three times a week, or once daily, to patients to maintain suitable serum levels of hGH. For patients chronically receiving hGH, the frequent injections result in patient compliance problems.

15 To resolve the problems associated with repetitive injections of aqueous hGH, attempts have been made to formulate controlled release devices containing higher doses of hGH than a bolus injection, encapsulated within a polymeric matrix wherein the hGH would be released *in vivo* over a period of about a week or more.

20 However, these controlled release devices often exhibited high initial bursts of hGH release and minimal hGH release thereafter. Further, due to the high concentration of hGH within these controlled release devices, the hGH molecules have tended to aggregate after 25 several days to form aggregated hGH which is immunogenic *in vivo* and likely has reduced biological activity.

Therefore, a need exists for a means for sustaining the release of biologically active hGH *in vivo* without causing an immune system response over the release period 30 of the hGH.

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Summary of the Invention

This invention relates to a composition, and methods of forming and using said composition, for the sustained release of biologically active, stabilized human growth 5 hormone (hGH). The sustained release composition of this invention comprises a polymeric matrix of a biocompatible polymer and particles of biologically active, metal cation-stabilized hGH, wherein said particles are dispersed within the biocompatible polymer.

10 The method of the invention for forming a composition for the sustained release of hGH, includes dissolving a biocompatible polymer in a polymer solvent to form a polymer solution, dispersing particles of biologically active, stabilized hGH in the polymer solution, and then 15 solidifying the polymer to form a polymeric matrix containing a dispersion of said hGH particles.

The method of using the sustained release composition of the present invention comprises providing a therapeutically effective blood level of biologically 20 active, non-aggregated human growth hormone in a subject for a sustained period by administering to the subject a dose of said sustained release composition.

The advantages of this sustained release formulation for hGH include longer, more consistent in vivo blood 25 levels of hGH, lower initial bursts of hGH, and increased therapeutic benefits by eliminating fluctuations in serum hGH levels. The advantages also include increased patient compliance and acceptance by reducing the required number of injections. The advantages further include the ability 30 to use smaller amounts of hGH compared to bolus injection regimen because serum hGH levels are maintained closer to therapeutical thresholds.

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Detailed Description of the Invention

The human growth hormone (hGH) used in this invention is biologically active hGH in its molecular (monomeric or non-aggregated) form. Molecular hGH is typically non-  
5 immunogenic.

Aggregated hGH may induce an immune response resulting in antibodies formed against hGH. This may compromise the efficacy of long-term hGH therapy. Additionally, aggregated hGH may stimulate an auto-immune response to  
10 endogenous hGH.

A sustained release of biologically active, non-aggregated human growth hormone is a release which results in measurable serum levels of biologically active, monomeric hGH over a period longer than that obtained  
15 following direct administration of aqueous hGH. It is preferred that a sustained release be a release of hGH for a period of about a week or more, and more preferably for a period of about two weeks or more.

A sustained release of biologically active, non-  
20 aggregated hGH from a polymeric matrix can be continuous or non-continuous release with relatively constant or varying rates of release. The continuity of hGH released and level of hGH released can be established by using, *inter alia*, one or more types of polymer compositions, hGH loadings,  
25 and/or selection of excipients to produce the desired effect.

Stabilized (hGH) comprises biologically active, non-aggregated hGH which is complexed with at least one type of multivalent metal cation, having a valency of +2 or more,  
30 from a metal cation component. Stabilized hGH in the sustained release composition of the present invention is in particulate form.

Suitable multivalent metal cations include metal cations contained in biocompatible metal cation components.  
35 A metal cation component is biocompatible if the cation component is non-toxic to the recipient, in the quantities used, and also presents no significant deleterious or

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untoward effects on the recipient's body, such as an immunological reaction at the injection site.

Typically, the molar ratio of metal cation component to hGH, for the metal cation stabilizing the hGH, is 5 between about 4:1 to about 10:1.

A preferred metal cation used to stabilize hGH is Zn<sup>+2</sup>. In a more preferred embodiment, the molar ratio of metal cation component, containing Zn<sup>+2</sup> cations, to hGH is about 6:1.

10 The suitability of a metal cation for stabilizing hGH can be determined by one of ordinary skill in the art by performing a variety of stability indicating techniques such as polyacrylamide gel electrophoresis, isoelectric focusing, reverse phase chromatography, HPLC and potency 15 tests on hGH lyophilized particles containing metal cations to determine the potency of the hGH after lyophilization and for the duration of release from microparticles. In stabilized hGH, the tendency of hGH to aggregate within a microparticle during hydration *in vivo* and/or to lose 20 biological activity or potency due to hydration or due to the process of forming a sustained release composition, or due to the chemical characteristics of a sustained release composition, is reduced by complexing at least one type of metal cation with hGH prior to contacting the hGH with a 25 polymer solution.

Stabilized hGH is typically stabilized against significant aggregation *in vivo* over the sustained release period. Significant aggregation is defined as an amount of aggregation resulting in aggregation of about 15% or more 30 of the initial amount of encapsulated hGH monomer. Preferably, aggregation is maintained below about 5% of the initial dose of hGH monomer. More preferably, aggregation is maintained below about 2% of the initial dose.

The hGH in a hGH sustained release composition can 35 also be mixed with other excipients, such as bulking agents or additional stabilizing agents, such as buffers to stabilize the hGH during lyophilization.

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Bulking agents typically comprise inert materials. Suitable bulking agents are known to those skilled in the art.

A polymer, or polymeric matrix, suitable for the 5 sustained release composition of the present invention, must be biocompatible. A polymer is biocompatible if the polymer, and any degradation products of the polymer, are non-toxic to the recipient and also present no significant deleterious or untoward effects on the recipient's body, 10 such as an immunological reaction at the injection site.

The polymer of the hGH sustained release composition must also be biodegradable. Biodegradable, as defined herein, means the composition will degrade or erode *in vivo* to form smaller chemical species. Degradation can result, 15 for example, by enzymatic, chemical and physical processes.

Suitable biocompatible, biodegradable polymers include, for example, poly(lactides), poly(glycolides), poly(lactide-co-glycolides), poly(lactic acid)s, poly(glycolic acid)s, poly(lactic acid-co-glycolic acid)s, 20 polycaprolactone, polycarbonates, polyesteramides, polyanhydrides, poly(amino acids), polyorthoesters, polycyanoacrylates, poly(p-dioxanone), poly(alkylene oxalate)s, biodegradable polyurethanes, blends and copolymers thereof.

25 Further, the terminal functionalities of the polymer can be modified. For example, polyesters can be blocked, unblocked or a blend of blocked and unblocked polymers. A blocked polymer is as classically defined in the art, specifically having blocked carboxyl end groups.

30 Generally, the blocking group is derived from the initiator of the polymerization and is typically an alkyl group. An unblocked polymer is as classically defined in the art, specifically having free carboxyl end groups.

Acceptable molecular weights for polymers used in this 35 invention can be determined by a person of ordinary skill in the art taking into consideration factors such as the desired polymer degradation rate, physical properties such

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as mechanical strength, and rate of dissolution of polymer in solvent. Typically, an acceptable range of molecular weights is of about 2,000 Daltons to about 2,000,000 Daltons. In a preferred embodiment, the polymer is a biodegradable polymer or copolymer. In a more preferred embodiment, the polymer is a poly(lactide-co-glycolide) (hereinafter "PLGA") with a lactide:glycolide ratio of about 1:1 and a molecular weight of about 5,000 Daltons to about 70,000 Daltons. In an even more preferred embodiment, the molecular weight of the PLGA used in the present invention has a molecular weight of about 6,000 to about 31,000 Daltons.

The amount of hGH, which is contained in a dose of sustained release microparticles, or in an alternate sustained release device, containing biologically active, stabilized hGH particles is a therapeutically or prophylactically effective amount, which can be determined by a person of ordinary skill in the art taking into consideration factors such as body weight, condition to be treated, type of polymer used, and release rate from the polymer.

In one embodiment, an hGH sustained release composition contains from about 0.01% (w/w) to about 50% (w/w) of biologically active, stabilized hGH particles. The amount of such hGH particles used will vary depending upon the desired effect of the hGH, the planned release levels, the times at which hGH should be released, and the time span over which the hGH will be released. A preferred range of hGH particle loading is between about 0.1% (w/w) to about 30% (w/w) hGH particles. A more preferred range of hGH particle loading is between about 0.1% (w/w) to about 20% (w/w) hGH particles. The most preferred loading of the biologically active, stabilized hGH particles is about 15% (w/w).

In another embodiment, a hGH sustained release composition also contains a second metal cation component, which is not contained in the stabilized hGH particles, and

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which is dispersed within the polymer. The second metal cation component preferably contains the same species of metal cation, as is contained in the stabilized hGH.

Alternately, the second metal cation component can contain 5 one or more different species of metal cation.

The second metal cation component acts to modulate the release of the hGH from the polymeric matrix of the sustained release composition, such as by acting as a reservoir of metal cations to further lengthen the period 10 of time over which the hGH is stabilized by a metal cation to enhance the stability of hGH in the composition.

A metal cation component used in modulating release typically contains at least one type of multivalent metal cation. Examples of second metal cation components 15 suitable to modulate hGH release, include, or contain, for instance,  $Mg(OH)_2$ ,  $MgCO_3$  (such as  $4MgCO_3 \cdot Mg(OH)_2 \cdot 5H_2O$ ),  $ZnCO_3$  (such as  $3Zn(OH)_2 \cdot 2ZnCO_3$ ),  $CaCO_3$ ,  $Zn_3(C_6H_5O_7)_2$ ,  $Mg(OAc)_2$ ,  $MgSO_4$ ,  $Zn(OAc)_2$ ,  $ZnSO_4$ ,  $ZnCl_2$ ,  $MgCl_2$  and 20  $Mg_3(C_6H_5O_7)_2$ . A suitable ratio of second metal cation component-to-polymer is between about 1:99 to about 1:2 by weight. The optimum ratio depends upon the polymer and the second metal cation component utilized.

A polymeric matrix containing a dispersed metal cation component to modulate the release of a biologically active 25 agent from the polymeric matrix is further described in co-pending U.S. Patent Application No. 08/237,057, filed May 3, 1994, and co-pending PCT Patent Application PCT/US95/05511, the teachings of which are incorporated herein by reference in their entirety.

30 The hGH sustained release composition of this invention can be formed into many shapes such as a film, a pellet, a cylinder, a disc or a microparticle. A microparticle, as defined herein, comprises a polymeric component having a diameter of less than about one 35 millimeter and having stabilized hGH particles dispersed therein. A microparticle can have a spherical, non-spherical or irregular shape. It is preferred that a

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microparticle be a microsphere. Typically, the microparticle will be of a size suitable for injection. A preferred size range for microparticles is from about 1 to about 180 microns in diameter.

- 5 In the method of this invention for forming a composition for the sustained release of biologically active, non-aggregated hGH, a suitable amount of particles of biologically active, stabilized hGH are dispersed in a polymer solution.
- 10 A suitable polymer solution contains between about 1% (w/w) and about 30% (w/w) of a suitable biocompatible polymer, wherein the biocompatible polymer is typically dissolved in a suitable polymer solvent. Preferably, a polymer solution contains about 2% (w/v) to about 20% (w/v)
- 15 polymer. A polymer solution containing 5% to about 10% (w/w) polymer is most preferred.

A suitable polymer solvent, as defined herein, is solvent in which the polymer is soluble but in which the stabilized hGH particles are substantially insoluble and

- 20 non-reactive. Examples of suitable polymer solvents include polar organic liquids, such as methylene chloride, chloroform, ethyl acetate and acetone.

To prepare biologically active, stabilized hGH particles, hGH is mixed in a suitable aqueous solvent with

- 25 at least one suitable metal cation component under pH conditions suitable for forming a complex of metal cation and hGH. Typically, the complexed hGH will be in the form of a cloudy precipitate, which is suspended in the solvent. However, the complexed hGH can also be in solution. In an
- 30 even more preferred embodiment, hGH is complexed with  $Zn^{+2}$ .

Suitable pH conditions to form a complex of hGH typically include pH values between about 7.0 and about 7.4. Suitable pH conditions are typically achieved through use of an aqueous buffer, such as sodium bicarbonate, as

- 35 the solvent.

Suitable solvents are those in which the hGH and the metal cation component are each at least slightly soluble,

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such as in an aqueous sodium bicarbonate buffer. For aqueous solvents, it is preferred that water used be either deionized water or water-for-injection (WFI).

It is understood that the hGH can be in a solid or a dissolved state, prior to being contacted with the metal cation component. It is also understood that the metal cation component can be in a solid or a dissolved state, prior to being contacted with the hGH. In a preferred embodiment, a buffered aqueous solution of hGH is mixed with an aqueous solution of the metal cation component.

Typically, the complexed hGH will be in the form of a cloudy precipitate, which is suspended in the solvent. However, the complexed hGH can also be in solution. In an even more preferred embodiment, hGH is complexed with  $Zn^{+2}$ .

The complexed hGH is then dried, such as by lyophilization, to form a particulate of stabilized hGH. The complexed hGH, which is suspended or in solution, can be bulk lyophilized or can be divided into smaller volumes which are then lyophilized. In a preferred embodiment, the complexed hGH suspension is micronized, such as by use of an ultrasonic nozzle, and then lyophilized to form stabilized hGH particles. Acceptable means to lyophilize the complexed hGH mixture include those known in the art.

Preferably, particles of stabilized hGH are between about 1 to about 6 micrometers in diameter. The hGH particles can be fragmented separately, as described in co-pending U.S. Patent Application No. 08/006,682, filed January 21, 1993, which describes a process for producing small particles of biologically active agents, which is incorporated herein in its entirety by reference. Alternately, the hGH particles can be fragmented after being added to a polymer solution, such as by means of an ultrasonic probe or ultrasonic nozzle.

In another embodiment, a second metal cation component, which is not contained in the stabilized hGH particles, is also dispersed within the polymer solution.

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It is understood that a second metal cation component and stabilized hGH can be dispersed into a polymer solution sequentially, in reverse order, intermittently, separately or through concurrent additions. Alternately, a polymer, a 5 second metal cation component and stabilized hGH and can be mixed into a polymer solvent sequentially, in reverse order, intermittently, separately or through concurrent additions.

The method for forming a composition for modulating 10 the release of a biologically active agent from a biodegradable polymer is further described in co-pending U.S. Patent Application No. 08/237,057.

In this method, the polymer solvent is then solidified to form a polymeric matrix containing a dispersion of 15 stabilized hGH particles.

One suitable method for forming an hGH sustained 20 release composition from a polymer solution is the solvent evaporation method described in U.S. Patent No. 3,737,337, issued to Schnoring et al., U.S. Patent No. 3,523,906, issued to Vranchen et al., U.S. Patent No. 3,691,090, 25 issued to Kitajima et al., or U.S. Patent No. 4,389,330, issued to Tice et al. Solvent evaporation is typically used as a method to form hGH sustained release microparticles.

25 In the solvent evaporation method, a polymer solution containing a stabilized hGH particle dispersion, is mixed in or agitated with a continuous phase, in which the polymer solvent is partially miscible, to form an emulsion. The continuous phase is usually an aqueous solvent.

30 Emulsifiers are often included in the continuous phase to stabilize the emulsion. The polymer solvent is then evaporated over a period of several hours or more, thereby solidifying the polymer to form a polymeric matrix having a dispersion of stabilized hGH particles contained therein.

35 A preferred method for forming hGH sustained release microparticles from a polymer solution is described in U.S. Patent No. 5,019,400, issued to Gombotz et al., and co-

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pending U.S. Patent Application No. 08/443,726, filed May 18, 1995, the teachings of which are incorporated herein by reference in their entirety. This method of microsphere formation, as compared to other methods, such 5 as phase separation, additionally reduces the amount of hGH required to produce a sustained release composition with a specific hGH content.

In this method, the polymer solution, containing the stabilized hGH particle dispersion, is processed to create 10 droplets, wherein at least a significant portion of the droplets contain polymer solution and the stabilized hGH particles. These droplets are then frozen by means suitable to form microparticles. Examples of means for processing the polymer solution dispersion to form droplets 15 include directing the dispersion through an ultrasonic nozzle, pressure nozzle, Rayleigh jet, or by other known means for creating droplets from a solution.

Means suitable for freezing droplets to form 20 microparticles include directing the droplets into or near a liquified gas, such as liquid argon and liquid nitrogen to form frozen microdroplets which are then separated from the liquid gas. The frozen microdroplets are then exposed to a liquid non-solvent, such as ethanol, or ethanol mixed with hexane or pentane.

25 The solvent in the frozen microdroplets is extracted as a solid and/or liquid into the non-solvent to form stabilized hGH containing microparticles. Mixing ethanol with other non-solvents, such as hexane or pentane, can increase the rate of solvent extraction, above that 30 achieved by ethanol alone, from certain polymers, such as poly(lactide-co-glycolide) polymers.

A wide range of sizes of hGH sustained release 35 microparticles can be made by varying the droplet size, for example, by changing the ultrasonic nozzle diameter. If very large microparticles are desired, the microparticles can be extruded through a syringe directly into the cold liquid. Increasing the viscosity of the polymer solution

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can also increase microparticle size. The size of the microparticles can be produced by this process, for example microparticles ranging from greater than about 1000 to about 1 micrometers in diameter.

5 Yet another method of forming an hGH sustained release composition, from a polymer solution, includes film casting, such as in a mold, to form a film or a shape. For instance, after putting the polymer solution containing a dispersion of stabilized hGH particles into a mold, the  
10 polymer solvent is then removed by means known in the art, or the temperature of the polymer solution is reduced, until a film or shape, with a consistent dry weight, is obtained. Film casting of a polymer solution, containing a biologically active agent, is further described in co-  
15 pending U.S. Patent Application No. 08/237,057, the teachings of which are incorporated herein by reference in their entirety.

It is believed that the release of the hGH can occur by two different mechanisms. The hGH can be released by  
20 diffusion through aqueous filled channels generated in the polymeric matrix, such as by the dissolution of the hGH or by voids created by the removal of the polymer's solvent during the synthesis of the sustained release composition.

A second mechanism is the release of hGH due to  
25 degradation of the polymer. The rate of degradation can be controlled by changing polymer properties that influence the rate of hydration of the polymer. These properties include, for instance, the ratio of different monomers, such as lactide and glycolide, comprising a polymer; the  
30 use of the L-isomer of a monomer instead of a racemic mixture; and the molecular weight of the polymer. These properties can affect hydrophilicity and crystallinity, which control the rate of hydration of the polymer.  
Hydrophilic excipients such as salts, carbohydrates and  
35 surfactants can also be incorporated to increase hydration and which can alter the rate of erosion of the polymer.

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By altering the properties of the polymer, the contributions of diffusion and/or polymer degradation to hGH release can be controlled. For example, increasing the glycolide content of a poly(lactide-co-glycolide) polymer 5 and decreasing the molecular weight of the polymer can enhance the hydrolysis of the polymer and thus, provides an increased hGH release from polymer erosion.

In addition, the rate of polymer hydrolysis is increased in non-neutral pH's. Therefore, an acidic or a 10 basic excipient can be added to the polymer solution, used to form the microsphere, to alter the polymer erosion rate.

The composition of this invention can be administered to a human, or other animal, by injection, implantation (e.g., subcutaneously, intramuscularly, intraperitoneally, 15 intracranially, intravaginally and intradermally), administration to mucosal membranes (e.g., intranasally or by means of a suppository), or in situ delivery (e.g. by enema or aerosol spray) to provide the desired dosage of hGH based on the known parameters for treatment with hGH of 20 the various medical conditions.

The invention will now be further and specifically described by the following examples.

Example 1

Formation of  $Zn^{+2}$ -Stabilized hGH

25 Human growth hormone (hGH), whose DNA sequence is described in U.S. Patent 4,898,830, issued to Goeddel et al., was used in this Example. The human growth hormone was stabilized by forming insoluble complexes with zinc.

30 The hGH was dissolved in samples of a 4 mM sodium bicarbonate buffer (pH 7.2) to form hGH solutions with a concentrations between 0.1 and 0.5 mM hGH. A 0.9 mM  $Zn^{+2}$  solution was prepared from deionized water and zinc acetate dihydrate and then was added to the hGH solutions to form  $Zn^{+2}$ -hGH complex. The pH of the  $Zn^{+2}$ -hGH complex was then 35 adjusted to between 7.0 and 7.4 by adding 1% acetic acid.

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A cloudy suspended precipitate, comprising Zn<sup>+2</sup>-stabilized hGH formed.

The suspension of Zn<sup>+2</sup>-stabilized hGH was then micronized using an ultrasonic nozzle (Type V1A; Sonics and 5 Materials, Danbury, CT) and sprayed into a polypropylene tub (17 cm diameter and 8 cm deep) containing liquid nitrogen to form frozen particles. The polypropylene tub was then placed into a -80 °C freezer until the liquid 10 nitrogen evaporated. The frozen particles, which contained Zn<sup>+2</sup>-stabilized hGH, were then lyophilized to form Zn<sup>+2</sup>-stabilized hGH particles.

Example 2

Preparation of PLGA Microspheres Containing Biologically Active, Aggregation-Stabilized hGH

15 Microspheres containing Zn<sup>+2</sup>-stabilized human growth hormone (hGH) were prepared from hydrophilic poly(lactide-co-glycolide) polymer RG502H having free carboxyl end groups (hereinafter "unblocked-PLGA") (50:50 PLGA, 9,300 Daltons; Boehringer Ingelheim Chemicals, Inc.) or a more 20 hydrophobic PLGA polymer having blocked carboxyl end groups (hereinafter "blocked-PLGA") (50:50 PLGA, 10,000 Daltons; Lot #115-56-1, Birmingham Polymers, Inc., Birmingham, AL).

The polymer was dissolved in methylene chloride at room temperature. The lyophilized hGH particles were added 25 to the polymer solution and zinc carbonate was also added. The mixture was then sonicated to give a homogeneous suspension. The suspension was atomized through a sonicating nozzle on to a bed of frozen ethanol, overlaid with liquid nitrogen. The vessel containing the 30 microspheres was stored at -80°C to extract the methylene chloride and then freeze-dried to give a free-flowing powder.

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Example 3  
Analysis of Encapsulated hGH Protein

The integrity of encapsulated hGH was determined by dissolving unhydrated microspheres into methylene chloride and acetone, collecting the protein, freeze-drying and re-constituting in HEPES buffer containing 10 mM EDTA. Appropriate controls were run to ensure that the extraction process did not affect the integrity of the protein.

The integrity of the encapsulated hGH was analyzed by measuring the percent of hGH monomer contained in the hGH after encapsulation through size exclusion chromatography (SEC).

The results of SEC analyses of the hGH integrity of hGH sustained release microspheres are provided below.

	Formulation (polymer; % Zinc Carbonate)	% Monomer (SEC)
15	31K unblocked; 6% ZnCO <sub>3</sub>	98.6
	31K unblocked; 6% ZnCO <sub>3</sub>	99.2
	31K unblocked; 3% ZnCO <sub>3</sub>	97.7
20	31K unblocked; 3% ZnCO <sub>3</sub>	97.8
	31K unblocked; 1% ZnCO <sub>3</sub>	97.6
	31K unblocked; 0% ZnCO <sub>3</sub>	97.8
	31K unblocked; 0% ZnCO <sub>3</sub>	97.1
25	10K blocked; 1% ZnCO <sub>3</sub>	98.2
	10K blocked; 1% ZnCO <sub>3</sub>	98.4
	8K unblocked; 0% ZnCO <sub>3</sub>	98.5
	10K blocked; 1% ZnCO <sub>3</sub>	98.4

The results showed that the encapsulation process did not cause aggregation of the protein. The yield percent protein recovered by the extraction procedure (relative to the amount measured by nitrogen content of the microspheres) ranged from about 40 to 98%. The variability

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is thought to be associated with loss of material during the transfer steps in the procedure and the extraction procedure is being modified to increase protein recovery.

Example 4

5       Determination of the Effect of Zinc Carbonate  
          on In vitro Release Kinetics

10       The microspheres were formed as described in Example 2 and contained 15% w/w hGH (6:1 Zn:hGH protein complex); 0%, 1%, 6%, 10% or 20% w/w zinc carbonate; and poly(lactide-co-glycolide) polymer.

15       In vitro release kinetics of the hGH sustained release microsphere formulations containing various concentrations of zinc carbonate were determined by suspending an aliquot (10 mg) of each type of microsphere in different 1.5 ml samples of HEPES buffer (50 mM Hepes, 10 mM KCl, 0.1% NaN3) pH 7.2 and then incubating at 37 °C. The amount of protein released was quantitated by sampling the buffer at 1, 3, 7, 10, 14, 21, 28 days after incubation and replenishing with fresh buffer after each sampling.

20       A curve of cumulative percent released (relative to initial hGH content in the starting mass of microspheres) versus time was plotted. Released protein samples from each time point were assayed for hGH monomer content by size exclusion chromatography.

25       Zinc carbonate is thought to act as a reservoir of zinc ions so that the formation of the Zn-hGH complex is favored and dissociation into soluble hGH disfavored. Because the aqueous solubility of zinc carbonate is low, the release of zinc ions from the reservoir is slow thus 30 modulating the solubility of the protein.

      The analysis found that in the absence of zinc carbonate, the rate of release of the encapsulated hGH was very rapid and all the protein was released in a very short period.

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Example 5

Assay for hGH After in Vivo Degradation  
of Blocked-PLGA Zn<sup>+2</sup>-Stabilized hGH Microspheres

Microspheres of blocked-PLGA, containing 15% w/w Zn<sup>+2</sup>-5 stabilized hGH and 0%, 6%, 10% or 20% ZnCO<sub>3</sub>, were formed by the method of Example 2. Groups of test rats were injected subcutaneously with 50 mg samples of the different hGH microspheres. The rats were sacrificed after 60 days and the skin sample were excised from the injection sites. The 10 excised skin samples were placed in 10% Neutral Buffered Formalin for at least 24 hours. They were then trimmed with a razor blade to remove excess skin and placed in PBS.

Tissue samples were processed by Pathology Associates, Inc. (Frederick, MD). The skin samples were embedded in 15 glycomethacrylate, sectioned and assayed for the presence of hGH using a HistoScan/LymphoScan Staining Kit (Product #24-408M; Accurate Chemical & Scientific Corp., Westbury, NY) according to the manufacturer's instructions. Tissue samples were scored for the presence or absence of staining 20 which was indicative of the presence or absence of hGH in the sample.

All skin samples, associated with hGH microsphere injections, tested positive for the presence of hGH thus indicating that the blocked-PLGA microspheres still 25 contained hGH after 60 days *in vivo*.

The method described in Example 2 was used to form microspheres by encapsulating 0% or 15% w/w hGH, in the form of Zn:hGH complex, and also 0%, 1% or 6% w/w ZnCO<sub>3</sub> salt, within blocked-PLGA and within unblocked-PLGA.

30 In vivo degradation of unblocked-PLGA microspheres versus blocked-PLGA microspheres were compared by injecting samples of microspheres into rats and then analyzing the microspheres remaining at the injection site at various times post-injection. Three rats were assayed at each time 35 point for each microsphere sample. On the day of administration of the microspheres, 750  $\mu$ l of vehicle (3%

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carboxymethyl cellulose (low viscosity) and 1% Tween-20 in saline) was added to vials containing  $50 \pm 1$  mg of microspheres. Immediately, the vials were shaken vigorously to form a suspension which was then aspirated 5 into a 1.0 cc syringe without a needle.

Rats (Sprague-Dawley males) were anesthetized with a halothane and oxygen mixture. The injection sites (intrascapular region) were shaven and marked with a permanent tattoo to provide for the precise excision of skin 10 at the sampling time points. Each rat was injected with an entire vial of microspheres using 18 to 21 gauge needles.

On designated days (days 15, 30, 59 and 90 post-injection for animals receiving blocked-PLGA microspheres, or days 7, 14, 21, 28 and 45 post-injection for animals 15 receiving unblocked-PLGA microspheres) the rats were sacrificed by asphyxiation with  $\text{CO}_2$  gas and the skin at the injection sites (including microspheres) was excised. Since the microspheres tended to clump at the injection sites, the presence or absence of microspheres was 20 determined visually.

The visual inspections found that the unblocked-PLGA microspheres degraded substantially faster than the blocked-PLGA microspheres, and that the addition of  $\text{ZnCO}_3$  to the blocked-PLGA substantially slowed polymeric 25 degradation. For example, in the rats injected with unblocked-PLGA microspheres containing 0% hGH and 0% or 1%  $\text{ZnCO}_3$ , no microspheres were visible on day 21. In addition, for rats injected with blocked-PLGA microspheres containing 0% hGH and 0%  $\text{ZnCO}_3$ , a few microspheres were 30 visible on day 60 and none were visible on day 90. Furthermore, for rats injected with blocked-PLGA microspheres containing 0% or 15% hGH and 6%  $\text{ZnCO}_3$ , microspheres were visible on day 90.

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Example 6

In Vivo Pharmacokinetic Studies  
of hGH Sustained Release Microspheres in Rats

Studies were conducted in rats to screen various hGH  
5 microsphere formulations, determine pharmacokinetic  
parameters following intravenous (IV), subcutaneous (SC)  
and SC osmotic pump (Alzet) administration of hGH, and to  
evaluate serum profiles and *in vivo* release-rate of various  
hGH microsphere formulations.

10 Sprague-Dawley rats were divided into groups of three  
each, randomized by body weight, and one hGH microsphere  
formulation was administered to each group. Rats were  
injected subcutaneously with approximately 7.5 mg of hGH in  
50 mg of one type of the different microspheres, suspended  
15 in 0.75 ml of an aqueous injection vehicle. The vehicle  
composition was 3% CMC (low viscosity), 1% Polysorbate 20,  
in 0.9% NaCl. The microsphere dose delivered was  
determined indirectly by weighing the residual dose in the  
injection vial and correcting for residual injection  
20 vehicle. The hGH dose was then computed from the protein  
loading of the microspheres determined by nitrogen  
analysis.

Blood samples were collected at pre-determined  
intervals for up to 30 days after injection. Blood samples  
25 of 250  $\mu$ l were collected during the first 24 hours and at  
least 400  $\mu$ l at time points after 24 hours. Blood samples  
were clotted and hGH concentrations in serum were  
determined using a radio-immuno assay. A radio-immunoassay  
(RIA) kit from ICN was validated and used to determine the  
30 hGH levels in rat serum.

For the determination of pharmacokinetic parameters,  
hGH in saline was administered to rats by subcutaneous  
bolus injection, intravenously or delivered via an osmotic  
pump (Alzet Model 2ML4) which was implanted subcutaneously.

35 Three groups of rats received single subcutaneous  
injections of hGH in 0.9% NaCl at 0.5 or 7.5 mg/kg at a

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dose volume of 1.0 ml/kg and two groups received single intravenous bolus injections of hGH in 0.9% NaCl solution at about 1.0 mg and 5.0 mg of hGH per kg rat with a dose volume of 1.0 ml/kg. For the Alzet pump study, rats were 5 divided into four groups of three rats each, randomized by body weight and dosed with about 20 mg/ml and 40 mg/ml hGH in 0.9% saline solution loaded into pumps (Alzet Model 2002, 200  $\mu$ l, 14 days release), and with about 4 mg/ml and 12 mg/ml hGH in 0.9% saline solution loaded into pumps 10 (Alzet Model 2ML4, 2ml, 28 days release). Expected release rates from the pumps correspond to about 2% and 4 to 6% of the ProRelease hGH dose (about 15 mg/kg) per day, respectively. The Alzet pumps were implanted 15 subcutaneously in the inter-scapular region after soaking for 1-2 minutes in sterile saline.

The formulations of hGH sustained release microspheres, synthesized as described in Example 2 contained 15% w/w hGH complexed with Zn in a ratio of 6:1 Zn:hGH; 0%, 1%, 3% or 6% w/w zinc carbonate; and 8K 20 unblocked PLGA, 10K blocked PLGA or 31K unblocked PLGA.

To evaluate the various hGH sustained release formulations, Cmax, Cd5 and Cmax/Cd5 were the in vivo 25 indices used, where Cmax is the maximum serum concentration observed, and Cd5 is the serum concentration at day 5 which should approximate the steady state concentration. The results were as follows:

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	Formulation	'Burst' in vitro (%)	% Monomer Day 7	Cmax (ng/ml)	C day 5 (ng/ml)	Cmax/Css
5	8K PLGA unblocked 0% ZnCO <sub>3</sub>	22.0 ± 0.9	99.3*	323.3 ± 98.6	20.4 ± 14.2	19.5 ± 10.6
	8K PLGA unblocked 1% ZnCO <sub>3</sub>	16.4 ± 1.6	97.3*	309.0 ± 67.1	20.4 ± 14.2	39.5 ± 17.7
	8K PLGA unblocked 3% ZnCO <sub>3</sub>	15.9 ± 6.9	98.7	670.5 ± 244.4	9.0 ± 4.2	44.8 ± 22.6
10	8K PLGA unblocked 6% ZnCO <sub>3</sub>	17.6 ± 2.7	99.3	358.0 ± 58.9	18.8 ± 14.7	42.4 ± 6.8
	31K PLGA unblocked 0% ZnCO <sub>3</sub>	12.3 ± 1.1	98.2	592 ± 318.2	4.5 ± 1.5	132.5 ± 47.9
	31K PLGA unblocked 1% ZnCO <sub>3</sub>	11.4 ± 1.3	98.8	432.7 ± 91.6	5.1 ± 0.3	84.1 ± 14.9
15	31K PLGA unblocked 3% ZnCO <sub>3</sub>	7.9 ± 1.9	99.4	643.6 ± 203.9	8.0 ± 2.6	93.3 ± 62.0
	31K PLGA unblocked 6% ZnCO <sub>3</sub>	15.8 ± 0.5	99.8	1691.8 340.0±	6.6 ± 0.8	262.2 ± 83.5
	10K PLGA blocked 1% ZnCO <sub>3</sub>	12.7 ± 0.1	99.3	615.9 ± 384.3	4.5 ± 1.0	155.0 ± 126.8
20	10K PLGA blocked 3% ZnCO <sub>3</sub>	18.1 ± 3.2	99.6	1053.2 ± 293.3	3.6 ± 0.8	291.7 ± 71.1
	10K PLGA blocked 6% ZnCO <sub>3</sub>	9.9 ± 1.4	99.0	1743.5 ± 428.4	4.9 ± 2.7	516.1 ± 361.6
25						
30						
35						
	*	Value obtained from duplicate batch of the same formulation.				

The results of the screening showed that the two unblocked polymers (8K and 31K) had different in vivo release kinetics compared to the original formulation,

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which used blocked 10K PLGA and 6% w/w zinc carbonate. Cmax values were generally lower with the unblocked polymer formulations than with the lead formulation which suggested that the in vivo 'burst' may be lower with the unblocked 5 polymer formulations. The 'burst' was defined as the percent of hGH released in the first 24 hours after injection. The in vitro 'burst' values were between 8-22%. The zinc carbonate content of the formulations did not appear to have an effect on the 'burst' or the in vitro 10 release profile.

The serum concentrations between days 4 and 6 were maintained at a fairly constant level above baseline (or the pre-bleed levels) with the unblocked polymer formulations, while serum concentrations with the blocked 15 formulations, at the same time points were close to the baseline levels. The in vitro release data for up to 7 days showed that the released hGH protein was monomeric. Useful data could not be obtained beyond day 6 because of anti-hGH antibody formulation in the rats.

20

Example 7  
Rhesus Monkey Pharmacokinetics Study

The objective of this primate study was to evaluate the pharmacokinetic profiles of different hGH sustained 25 release formulations as compared to more traditional methods of administering hGH (e.g., bolus sc injections, daily sc injections and sc injection combined with the use of an osmotic pump) and to determine which hGH sustained release formulation gave the optimal hGH blood concentration profile

30 The formulations for the hGH sustained release microspheres tested were 1) 15% hGH (complexed with Zn at a 6:1 Zn:hGH ratio), 6% w/w zinc carbonate and 10K blocked PLGA; 2) 15% hGH (complexed with Zn at a 6:1 Zn:hGH ratio), 1% w/w zinc carbonate and 8K unblocked PLGA ("RG502H" PLGA 35 polymer); and 3) 15% hGH (complexed with Zn at a 6:1 Zn:hGH

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ratio), 1% w/w zinc carbonate and 31K unblocked PLGA ("RG503H" PLGA polymer).

There were four monkeys per group and each animal received a single subcutaneous injection into the dorsal cervical region on Day 1. A dose of 160 mg of hGH sustained release microspheres (24 mg of hGH) was administered to each monkey in 1.2 ml of injection vehicle through a 20 gauge needle. The injection vehicle was an aqueous vehicle containing 3% w/v Carboxymethyl Cellulose (sodium salt), 1% v/v Tween 20 (Polysorbate 20) and 0.9% sodium chloride.

The hGH dose was intended to provide measurable hGH serum concentrations for pharmacokinetic analysis. To obtain pharmacokinetic parameters additional study groups of four monkeys each were included, specifically 1) a single subcutaneous injection (24 mg hGH), 2) daily subcutaneous injections (24 mg/28 days = 0.86 mg hGH/day), 3) a subcutaneous injection (3.6 mg hGH) combined with an Alzet osmotic pump (20.4 mg hGH) (total dose of 24 mg hGH), and 4) a subcutaneous injection of the injection vehicle as a control (only used 3 monkeys for the vehicle control group).

Blood samples were collected at the following times for hGH, IGF1, IGFBP3 and anti-hGH antibody analyses: -7, -5, -3, pre-dose and, 0.5, 1, 2, 3, 5, 8, 10, 12, 24, 28, 32 and 48 hours, 5, 4, 6, 8, 11, 14, 17, 20, 23, 26, 29, 32, 25, 28, 41, 44, 47, 50, 53, 56 days post-dose.

The concentrations of IGF-1 and hGH in the serum were then measured. An IRMA kit from RADIM (distributed by: Wein Laboratories, P.O. Box 227, Succasunna, NJ) was used to quantify hGH in monkey serum. The IRMA assay had a limit of quantification in PBS buffer of 0.1 ng/mL and in pooled juvenile rhesus monkey serum of 1.5 ng/mL with a basal GH level of about 4ng/mL.

The IRMA assay was validated over the concentration range 1.5 - 75 ng/mL for pooled juvenile rhesus monkey

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serum. The measurement precision and accuracy are within the range of  $\pm 10\%$ .

The results showed that the hGH sustained release microspheres were releasing significant, sustained levels 5 of hGH over a one month period while the subcutaneous injections were not able to maintain the same serum levels.

The IGF-1 serum profile showed that serum IGF-1 concentrations were elevated above the baseline values between days 2 and 29 after administering the 10 microparticles. This shows that enough hGH was being released from the hGH sustained release microspheres to cause a pharmacodynamic effect. This also indicates that the hGH released was biologically active which suggest that the encapsulation process had not adversely affected the 15 biopotency of hGH.

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to specific embodiments of the invention 20 described specifically herein. Such equivalents are intended to be encompassed in the scope of the following claims.

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CLAIMS

The invention claimed is:

1. A composition for the sustained release of human growth hormone comprising:
  - 5 a) a biocompatible polymer; and
  - b) particles of metal cation-stabilized human growth hormone, wherein the metal cation is Zn(II).
2. A composition for the sustained release of human growth hormone comprising:
  - 10 a) a biocompatible polymer; and
  - b) particles of metal cation-stabilized human growth hormone, wherein the molar ratio of metal cation to protein is between about 4:1 and 10:1, more preferably at least about 6:1.
- 15 3. The composition of Claim 2 wherein the metal cation is Zn(II).
4. The composition of any one of the preceding claims wherein the metal cation is added to human growth hormone as a water soluble salt.
- 20 5. The composition of Claim 4 wherein the metal cation is added to human growth hormone in the form of zinc acetate.
- 25 6. The composition of any one of the preceding claims wherein said particles of metal cation-stabilized human growth hormone are dispersed within said biocompatible polymer.

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7. The composition of Claim 6 wherein said polymer is selected from the group consisting of poly(lactide), poly(glycolide), poly(lactide-co-glycolide), poly(lactic acid), poly(glycolic acid),  
5 polycaprolactone, polycarbonate, polyesteramide, polyanhydride, poly(amino acid), polyorthoester, polycyanoacrylate, poly(dioxanone), poly(alkylene oxalate), polyurethane, blends and copolymers thereof.
8. The composition of Claim 7 wherein said polymer is  
10 selected from the group consisting of poly(lactide), poly(glycolide) and poly(lactide-co-glycolide).
9. The composition of any one of the preceding claims wherein the particles of metal cation-stabilized human growth hormone is present in said polymer at a  
15 concentration between about 0.1% and about 30% by weight, preferably between about 0.1% and about 20% by weight.
10. The composition of Claim 9 wherein the particles of metal cation-stabilized human growth hormone is present in said polymer at a concentration of about  
20 15% by weight.
11. The composition of any one of the preceding claims wherein the biocompatible polymer further comprises a metal cation component.
- 25 12. The composition of Claim 11 wherein the metal cation of the metal cation component is Zn(II).
13. The composition of Claim 12 wherein the metal cation component is selected from the group consisting of a  $ZnCO_3$ ,  $Zn_3(C_6H_5O_7)_2$ ,  $Zn(OAc)_2$ ,  $ZnSO_4$  and  $ZnCl_2$ .

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14. The composition of Claim 11 wherein the metal cation of the metal cation component is Mg(II).
15. The composition of Claim 12 wherein the metal cation component is selected from the group consisting of a  
5  $Mg(OH)_2$ ,  $MgCO_3$ ,  $Mg_3(C_6H_5O_7)_2$ ,  $Mg(OAc)_2$ ,  $MgSO_4$  and  $MgCl_2$ .
16. The composition of any one of Claims 12-15 wherein the ratio of the metal cation component to polymer is between about 1:99 to about 1:2 by weight, preferably at about 1% by weight.
- 10 17. The composition of Claim 1 for the sustained release of human growth hormone comprising:
  - a) a biocompatible poly(lactide-co-glycolide) having dispersed therein Zn(II) wherein the Zn(II) is added as  $ZnCO_3$ ; and
  - 15 b) particles of Zn(II)-stabilized human growth hormone (hGH), wherein the Zn(II) is added to the human growth hormone in the form of zinc acetate at a molar ratio of about 6:1 Zn:hGH; and the particles of Zn(II)-stabilized human growth  
20 hormone is present in said polymer at a concentration of about 15% by weight.
18. The composition of any one of the preceding claims for use in the preparation of a medicament for therapy.

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(54) Title: COMPOSITION FOR SUSTAINED RELEASE OF HUMAN GROWTH HORMONE

(57) Abstract

A composition, and methods of forming and using said composition, for the sustained release of biologically active, stabilized human growth hormone (hGH). The sustained release composition of this invention comprises a polymeric matrix of a biocompatible polymer and particles of biologically active, stabilized hGH, wherein said particles are dispersed within the biocompatible polymer. The method of the invention for producing a composition for the sustained release of biologically active hGH, includes dissolving a biocompatible polymer in a polymer solvent to form a polymer solution, dispersing particles of biologically active, metal cation-stabilized hGH in the polymer solution, and then solidifying the polymer to form a polymeric matrix containing a dispersion of said hGH particles. The method for using a composition of the invention is a method for providing a therapeutically effective blood level of biologically active, non-aggregated hGH in a subject for a sustained period. In this method, a subject is administered an effective dose of the sustained release composition of the present invention. The method of using the sustained release composition of the present invention comprises providing a therapeutically effective blood level of biologically active, non-aggregated human growth hormone in a subject for a sustained period by administering to the subject a dose of said sustained release composition.

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## INTERNATIONAL SEARCH REPORT

International Application No
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A. CLASSIFICATION OF SUBJECT MATTER		
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According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>WO,A,94 12158 (ALKERMES) 9 June 1994</p> <p>see page 13 - page 14; examples 1,2</p> <p>see page 9, paragraph 3</p> <p>see page 11, paragraph 5 - page 12, paragraph 3</p> <p>---</p>	1-4, 6-13,15, 16,18
P,X	<p>WO,A,95 29664 (ALKERMES) 9 November 1995</p> <p>see claim 1</p> <p>see page 34; example 12</p> <p>see page 10, line 29 - page 11, line 14</p> <p>---</p> <p>-/-</p>	1,6-16, 18

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Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
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European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Ventura Amat, A

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## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO,A,96 07399 (TAKEDA CHEMICAL INDUSTRIES) 14 March 1996 see claims 1-5,11,16,17 see page 8, line 4 - line 17 see page 9, line 35 - page 10, line 10 see page 11, line 17 - page 12, line 9 see page 23; example 3 see page 27; example 4 -----	1,4-10, 18

1

**INTERNATIONAL SEARCH REPORT**

Information on patent family members

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<b>PCT/US 96/08086</b>					
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